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(54) Title: THREE COMPONENT CHIMERIC ANTISENSE OLIGONUCLEOTIDES

(57) Abstract

The present application describes a novel family of oligonucleotide compounds having a novel organization of various modified nucleotides and modified chemical linkages. The application further discloses that limiting the presence and extent of specific modified nucleotides/modified linkages in the oligomers results in enhanced activation of endogenous RNase H activity.

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THREE COMPONENT CHIMERIC ANTISENSE OLIGONUCLEOTIDES

This application claims priority to U.S. Applications Nos. 60/026,732 filed September 26, 1996, and 08/754,580 filed November 21, 1996.

1. FIELD OF THE INVENTION

This invention relates to antisense oligonucleotides that target mRNAs in cells as substrates for the cellular enzyme RNase H and thereby cause specific degradation of the targeted mRNA. The oligonucleotides have four components: an RNase H activating region; a complementarity region; a 5' end; and a 3' end. The invention optimizes each of the components to resist intracellular nucleases, to increase hybridization to target mRNA, to specifically inactivate target mRNA in cells, and to decrease cytotoxicity.

2. BACKGROUND TO THE INVENTION

Antisense polynucleotides are useful for specifically inhibiting unwanted gene expression in mammalian cells. They can be used to hybridize to and inhibit the function of an RNA molecule, typically a messenger RNA, by activating RNase H.

The use of antisense oligonucleotides has emerged as a powerful new approach for the treatment of certain diseases. The preponderance of the work to date has focused on the use of antisense oligonucleotides as antiviral agents or as anticancer agents (Wickstrom, E., Ed., Prospects for Antisense Nucleic Acid Therapy of Cancer and AIDS, New York: Wiley-Liss, 1991; Crooke, S.T. and Lebleu, B., Eds., Antisense Research and Applications, Boca Raton: CRC Press, 1993, pp. 154-182; Baserga, R. and Denhardt, D.T., 1992, Antisense Strategies, New York: The New York Academy of Sciences, Vol. 660; Murray, J.A.H., Ed., Antisense RNA and DNA, New York: Wiley-Liss, 1993).

There have been numerous disclosures of the use of antisense oligonucleotides as antiviral agents. For example, Agrawal *et al.* report phosphoramidate and phosphorothioate

oligonucleotides as antisense inhibitors of HIV. Agrawal et al., Proc. Natl. Acad. Sci. USA 85:7079-7083 (1988). Zamecnik et al. disclose antisense oligonucleotides as inhibitors of Rous sarcoma virus replication in chicken fibroblasts. Zamecnik et al., Proc. Natl. Acad. Sci. USA 83: 4143-4146 (1986).

The principal mechanism by which antisense oligonucleotides affect a targeted RNA molecule is by activation of the cellular enzyme RNase H, which cleaves the RNA strand of DNA/RNA hybrids. Both phosphodiester and phosphorothioate-linked DNA activates endogenous RNase H, thereby cleaving the targeted RNA (Agrawal, S., et al., Proc. Natl. Acad. Sci. USA 87:1101-5 (1990); Woolf, T.M., et al., Nucleic Acids Res. 18:1763-9 (1990)). However, phosphodiester-linked DNA is rapidly degraded by cellular nucleases and, with the exception of the phosphorothioate-linked DNA, nuclease resistant, non-naturally occurring DNA derivatives do not activate RNase H when hybridized to RNA. While phosphorothioate DNA has the advantage of activating RNase H, phosphorothioate-linked DNA has been associated with non-specific cytotoxic effects and a reduced affinity for RNA (Stein, C.A., et al., Aids Res Hum Retroviruses 5:639-46 (1989); Woolf, T.M., et al., Nucleic Acids Res. 18:1763-9 (1990); Kawasaki, A.M., et al., J. Med. Chem. 36:831-41 (1993)).

Chimeric antisense oligos that have a short stretch of phosphorothioate DNA (3-9 bases) have been used to obtain RNase H-mediated cleavage of the target RNA (Dagle, J.M., et al., Nucleic Acids Res. 18:4751-7 (1990); Agrawal, S., et al., Proc. Natl. Acad. Sci. USA 87:1401-5 (1990); Monia, B.P. et al., 1993, J. Biol. Chem. 268:14514) A minimum of 3 DNA bases is required for activation of bacterial RNase H (Futdon, P.J., et al., Nucleic Acids Res. 17:9193-9204; Quartin, R.S., et al., Nucleic Acids Res. 17:7235-7262) and a minimum of 5 bases is required for activation of mammalian RNase H (Monia, B.P., et al., J. Biol. Chem. 268:14514-14522 (1993)). In these chimeric oligonucleotides there is a

central region that forms a substrate for RNase H that is flanked by hybridizing "arms," comprised of modified nucleotides that do not form substrates for RNase H. Alternatively, extracellular tests using a RNase H-containing HeLa cell extract have been reported wherein the RNase H activating region was placed on the 5' or 3' side of the oligomer. Specifically, these tests reported that a 5' or 3' terminal RNase H activating region composed of phosphodiester 2'-deoxynucleotides joined to a methylphosphonate-linked complementarity region was fully active, but that a 5' terminal RNase H-activating region composed of phosphorothioate 2'-deoxynucleotides joined to a methylphosphonate-linked complementarity region was only partially active. See Col 10, U.S. Pat. No. 5,220,007 to T. Pederson *et al.*.

2'-O-Methyl or 2'-fluoro modified nucleotides have been used for the hybridizing arms of chimeric oligos. Inoue, H., *et al.*, 1987, Nucleic Acids Res. 15:6131-48. The 2'-O-Methyl group increases the affinity of the oligomer for the targeted RNA and increases the activity of the oligomer in cell culture. However, 2'-O-Methyl bases with phosphodiester linkages are degraded by exonucleases and so are not suitable for use in cell or therapeutic applications of antisense. Shibahara, S., *et al.*, 1989, Nucleic Acids Res. 17:239-52. Phosphorothioate 2'-O-Methyl nucleotides are resistant to nucleases as shown in the uniformly phosphorothioate modified oligos described by Monia B.P., *et al.*, 1993, J. Biol. Chem. 268:14514-14522 and terminal phosphorothioate substituted, 2'-O-Methylribo-oligonucleotides, Shibahara, S., *et al.*, 1989, Nucleic Acid Res. 17:239-252. However, fully phosphorothioate substituted oligomers may cause non-specific effects including cell toxicity. Stein, C.A., *et al.*, 1989, Aids Res. Hum. Retrov. 5:639-646; Woolf, T.M., *et al.*, 1990, Nucleic Acids Res. 18:1763-69; Wagner, R.W., 1995, Antisense Res. Dev. 5:113-115; Krieg, A.M., & Stein, C.A., 1995, Antisense Res. Dev. 5:241.

The effects of 2'-Fluoro-oligonucleotides on bacterial RNase H are discussed in Crooke, S.T. *et al.*, 1995, Bioch. J. 312:599-608 and Iwai, S. *et al.*, 1995, FEBS Lett (Neth.) 368:315-20.

Several other chemistries have been used to make the "arms" or regions of a chimeric oligomer that are not substrates for RNase H. The first chimeric oligomers used methylphosphonate or phosphoramidate linkages in the arms (Dagle, J.M., Walder, J.A. & Weeks, K.L., Nucleic Acids Res. 18:1751-7 (1990); Agrawal, S., *et al.*, Proc. Natl. Acad. Sci. USA 87:1401-5 (1990). While these compounds functioned well in buffered systems and Xenopus oocytes, the arms decreased the hybrid affinity. This decrease in affinity dramatically reduced the antisense activity of the chimeric oligomers in mammalian cell culture.

A number of studies have been reported for the synthesis of ethylated and methylated phosphotriester oligonucleotides and their physico-chemical and biochemical evaluation. Dinucleotides with methyl and ethyl triesters were shown to possess greater affinity for polynucleotides possessing complementary sequences (Miller, P.S., *et al.*, J. Am. Chem. Soc. 93:6657, (1971)). However, a few years ago, another group reported lack of, or relatively poor, binding affinity of a heptaethyl ester of oligothymidine with complementary polynucleotides (Pless, R.C., and Ts'O, P.O.P., Biochemistry 16:1239-1250 (1977)). Phosphate methylated (P-methoxy) oligonucleotides were synthesized and found to possess resistance towards endonuclease digestion (Gallo, K.L., *et al.* Nucl. Acid Res. 18:7405 (1986)). A P-methoxy 18-mer oligonucleotide was shown to have high Tm value in duplexes with natural DNA and blocked the DNA replication process at room temperature (Moody, H.M., *et al.*, Nucl. Acid Res. 17:4769-4782 (1989)). Moody *et al.* concluded that phosphate ethylated (P-ethoxy) oligonucleotides would have poor antisense properties.

P-methoxy dimers of DNA bases were synthesized using FMOC as transient protecting group for the exocyclic amino

groups (Koole, L.H., *et al.*, J. Org. Chem. 54:1657-1664 (1989)). The synthesis and physico-chemical properties of partial P-methoxy oligodeoxyribonucleotides were also determined. Only thymidine and cytidine oligomers with methyl phosphotriester could be prepared because of the difficulties encountered in maintaining methyl triester intact. Furthermore, the methyl group was found to have destabilizing effect on the hybridization properties of the modified oligomers with their complementary sequences as compared to the unmodified parent oligodeoxyribonucleotide (Vinogradeov, S., Asseline, U., Thoung, N.T., Tet. Let. 34:5899-5902 (1993)).

Other reports have suggested that P-methoxy oligonucleotides are preferable to P-ethoxy as antisense oligonucleotides because of p-methoxy oligonucleotides showed stronger hybridization than methyl phosphonate or P-ethoxy oligonucleotides (van Genderen, M.H.P., *et al.*, Kon. Ned. Akad. van Wetensch. B90:155-159 (1987); van Genderen, M.H.P., *et al.*, Trav. Chim. Pays Bas 108:28-35 (1989)). P-ethoxy oligonucleotides were reported by van Genderen *et al.* to hybridize poorly to DNA and were thus deemed less suitable for use as antisense oligonucleotides (Moody, H.M., *et al.*, Nucl. Acid Res. 17:4769-4782 (1989)).

P-isopropoxyphosphoramidites have been synthesized from several nucleosides (Stec, W.J., *et al.*, Tet. Let. 26:2191-2194 (1985)), and a few short oligonucleotides containing P-isopropoxyphosphotriesters were synthesized, and hybridization studies were carried out.

United States Patent No. 5,525,719 to Srivastava, S., and Raza, S.K., June 11, 1996, suggests antisense oligonucleotides consisting of 2'-O-Methyl nucleotides linked by phosphodiester and/or P-ethoxy or P-methoxy, phosphotriester moieties.

Presently there are no nucleic acid chemistries nor any chimeras that have been developed that optimally achieve all the features that are needed to provide an effective antisense oligonucleotide i.e. low toxicity, high

specificity, nuclease resistance, ease of synthesis, RNase H compatibility.

3. SUMMARY OF THE INVENTION

The present invention describes a class of oligonucleotides that has been optimized to target a specific RNA target for RNase H degradation while remaining resistant to nuclease degradation in plasma and within eukaryotic, especially mammalian cells. The oligonucleotides of the invention contain no naturally occurring 5'→3'-linked nucleotides. Rather, the invention provides oligonucleotides having two types of nucleotides: 2'-deoxyphosphorothioate, which activate RNase H, and 2'-modified nucleotides, which do not. The linkages between the 2'-modified nucleotides can be phosphodiesters, phosphorothioate or P-ethoxyphosphodiester.

In addition to 5' and 3' ends, the presently described oligonucleotides comprise an RNase H activating region, and a complementarity region that facilitates hybridization to the target sequence. The RNase H-activating region is typically a contiguous sequence that contains between three and five 2'-deoxyphosphorothioate nucleotides (to activate bacterial RNase H), and typically between about 3 to 12, more typically 5 and 12, and more preferably between about 5 and 10 2'-deoxyphosphorothioate nucleotides to activate eukaryotic, particularly mammalian, RNase H.

The 5' and 3' ends of the presently described oligonucleotides are protected from exonuclease degradation via the incorporation of modified 5' and 3' terminal bases that are highly nuclease, particularly exonuclease, resistant and, optionally, by placing a 3' terminal blocking group.

In a preferred embodiment the RNase H activating region, is composed of highly nuclease resistant phosphorothioate nucleotides that is placed at the 5' end of the oligonucleotide.

Accordingly, one embodiment of the present invention is a chimeric oligonucleotide comprising an RNase H-activating region of between three and twelve contiguous

2'-deoxyphosphorothioate-linked bases (i.e., phosphorothioate linked 2'-deoxyribonucleotides); a substantially endonuclease resistant complementarity region of between about nine and about fifty 2'-modified bases; a substantially exonuclease resistant 5' terminus; and a substantially exonuclease resistant 3' terminus.

4. DETAILED DESCRIPTION OF THE INVENTION

4.1. THE STRUCTURE OF THE OLIGONUCLEOTIDES

An oligonucleotides of the presently invention typically comprise a 5' exonuclease resistant 5' terminal nucleic acid or linkage, a contiguous RNase H activating region of about 3 to about ten bases in length, a 3'-terminal 5'→3'-linked, or optionally 3'-3' linked, e.g., "inverted", nucleoside, and from about 9 to about 50 5'→3' linked nucleotides, which nucleotides can be 2'-deoxynucleotides or 2'-modified nucleotides that facilitate hybridization of the oligonucleotide to the target mRNA, such as 2'-fluoro, 2'-methoxy, 2'-ethoxy, 2'-methoxyethoxy, 2'-allyloxy (-$OCH_2CH=CH_2$) nucleotides (hereinafter "2'-modified nucleotides"). The 3' terminal nucleoside can, optionally, be a 2'-modified nucleoside. Those skilled in the art appreciate that the 3'-OH of the 3' terminal base can, but need not, be esterified to a phosphate or phosphate analog. The 3' terminal residue is referred to as a nucleoside even though it may be a nucleotide.

The internucleotide linkages of an oligonucleotide of the invention can be phosphodiester, phosphorothioate or P-ethoxyphosphodiester moieties. The oligonucleotide has a 3' terminus and a 5' terminus that are substantially protected from nuclease attack. The 3' terminus is protected by having the 3' most 5'→3' linkage or linkages be a phosphorothioate or a P-alkyloxyphosphotriester linkage and/or by having a substituted 3' terminal hydroxyl, e.g., a 3'→3' linked nucleotide, wherein the alkyloxy radical is methoxy, ethoxy or isopropoxy and, preferably, ethoxy. Preferably two or three 3' terminal internucleotide linkages are

phosphorothioate or a P-alkyloxyphosphotriester linkages. To reduce nuclease degradation, the 5' most 3'→5' linkage preferably should be a phosphorothioate linkage or P-alkyloxyphosphotriester linkage. Preferably, the two 5' most 3'→5' linkages should be phosphorothioate linkages or P-ethoxyphosphotriester linkages. Optionally, the 5'-terminal hydroxyl moiety can be esterified with a phosphorus containing moiety, e.g., phosphate, phosphorothioate or P-ethoxyphosphate, without limitation.

The 3' terminal 5'→3'-linked nucleoside has a 3'-O that can be optionally substituted by a blocking moiety that prevents 3'-exonuclease degradation of the oligonucleotide. In one embodiment, the 3'-hydroxyl is esterified to a nucleotide through a 3'→3' internucleotide linkage. Optionally, the 3'→3' linked nucleotide at the 3' terminus can be linked by a phosphorothioate moiety. By incorporating the above chemistries, the presently described oligonucleotides are substantially resistant to 5' and 3' exonucleases and endonucleases. For the purposes of the present invention, an oligomer is substantially resistant to a given endo or exonuclease when it is at least about 3-fold more resistant to attack by an endogenous cellular nuclease, and is highly nuclease resistant when it is at least about 6-fold more resistant than a corresponding oligomer comprised of unmodified DNA or RNA.

In a preferred embodiment, the oligonucleotide contains, exclusive of an optional blocking nucleotide, between 15 and 50 bases and more preferably between 20 and 30 bases and in a most preferred embodiment the oligonucleotide is 25 bases in length. The oligonucleotide of the invention contains a single contiguous RNase H-activating region of between three to ten 2'-deoxyphosphorothioate nucleotides. The length of the RNase H activating region to activate bacterial RNase H is preferably between three and five nucleotides; to activate a eukaryotic RNase H the activating region is preferably between about five and about ten or twelve nucleotides. The

preferred length of the RNase H-activating region for the activation of mammalian RNase H is nine nucleotides.

All 5'→3' linked nucleotides of the oligonucleotide that are not a part of the RNase H-activating region are 2'-modified nucleotides, which contribute to the target binding and thus form the complementarity determining region. The complementarity region can be a contiguous region or can be divided by the RNase H-activating region. In a preferred embodiment the complementarity region is a contiguous region, and, more preferably, is located 3' to the RNase H-activating region.

In a preferred embodiment all bases except for the one to three 3'-terminal nucleotides and/or nucleoside, the 5' terminal nucleotide, and the RNase H activating region nucleotides, are phosphodiester linked. Large amounts of contiguous phosphorothioate linkages are detrimental to the function of the oligonucleotides of the invention. Accordingly, the oligonucleotides preferably contain not more than twelve contiguous phosphorothioate linkages or twelve contiguous phosphorothioate linked deoxynucleotides.

Additional embodiments of the presently described chimeric oligonucleotides have the structures:

5' A:B:C

or

5' C:B:A:B:C.

Wherein A is a RNase H activating region of between about 3 to about 12 nucleotides, preferably about 3 to about 10 nucleotides or 5 to about 12 nucleotides long that is also nuclease stable (e.g., phosphorothioate DNA); B represents a region of chemistry (e.g. 2'O-methyl substituted RNA) that is stable against endonucleases (about 4 to about 40 nucleotides long; and C represents a one to four nucleotide long exonuclease block that typically does not contain phosphorothioate DNA (i.e., phosphorothioate 2'-O-methyl linkages, inverted bases, methylphosphonate, phosphoramidite, non-nucleotide linkers, amino linkers, conjugates or any

other chemistry consistent with nucleotide synthesis in the art, or yet to discovered that is not recognized by cellular exonucleases). Alternatively, the configuration may be inverted as follows:

5' C:B:A.

If the application does not require activation of RNase H (stearic blocking or triple strand inactivation), the following configuration is useful:

5' C:B:C.

4.2. SYNTHESIS OF THE OLIGONUCLEOTIDES

The oligonucleotides of the invention can be synthesized by solid phase or liquid phase nucleotide synthesis, however, synthesis by solid phase techniques is preferred. Phosphodiester and phosphorothioate linked oligonucleotides can be synthesized, using standard reagents and protocols, on an automated synthesizer utilizing methods that are well known in the art, such as, for example, those disclosed in Stec et al., J. Am. Chem. Soc. 106:6077-6089 (1984); Stec et al., J. Org. Chem. 50(20):3908-3913 (1985); Stec et al., J. Chromatog. 326:263-280 (1985); LaPlanche et al., Nuc. Acid. Res. 14:9081-9093 (1986); and Fasman, G.D., Practical Handbook of Biochemistry and Molecular Biology 1989, CRC Press, Boca Raton, Florida, herein incorporated by reference.

The synthesis of 2'-O-alkyl-oligoribonucleotides, where the alkyl groups are methyl, butyl, allyl or 3,3-dimethylallyl is reviewed by Lamond, Biochem. Soc. Trans. 21:1-8 (1993). Intermediates that are useful in the synthesis of 2'-O-methyl oligoribonucleotides are described in U.S. Patents No. 5,013,830, No. 5,525,719 and No. 5,214,135, which are hereby incorporated by reference.

The synthesis of 2'-fluorophosphodiester and 2'-fluorophosphorothioate oligonucleotides can be performed according to teaching of Kawasaki, A.M., et al., 1993. J. Med. Chem. 36:831-41 and WO 92/03568; the synthesis of P-alkyloxyphosphotriester-linked oligonucleotides and 2'-

modified oligonucleotides can be performed according to U.S. Patent No. 5,525,719, each of which is incorporated herein by reference. The synthesis of phosphorothioate oligodeoxynucleotides is taught by U.S. Patent No. 5,276,019 and No. 5,264,423, which is hereby incorporated by reference. Synthesis of 2'-substituted oligonucleotides can be performed by variations on the techniques disclosed therein.

The synthesis of the oligonucleotides of the invention must be conducted with great attention to quality control. It is particularly important that the phosphorothioate linkages not be contaminated with phosphodiester linkages. It is advisable to pre-test the individual reagent lots to ascertain that high coupling efficiency can be obtained therewith and to exercise all possible precautions to maintain anhydrous conditions.

The quality of the synthesis of oligonucleotides can be verified by testing the oligonucleotides by capillary electrophoresis and denaturing strong anion HPLC (SAX-HPLC). The method of Bergot & Egan, 1992, J. Chrom. 599:35-42 is suitable. SAX-HPLC is particularly useful to verify that the phosphorothioate nucleotides are completely thiolated, i.e., are not contaminated by a small percentage of phosphodiesters.

The synthesis of oligonucleotides having both phosphodiester and phosphorothioate linkages is associated with a side reaction whereby the phosphorothioate linkages are oxidized by the standard I_2 treatments that are used to oxidize the cyanoethyl phosphoramidite. This problem can be minimized but not eliminated by reducing the concentration or I_2 to as low as 0.001 M. Therefore, in a preferred embodiment, all phosphorothioates of the oligonucleotides of the invention are found at the 5'-end, so that no phosphorothioate bond is exposed to I_2.

4.3. THE USES OF THE OLIGONUCLEOTIDES

The oligonucleotides of the invention can be used as antisense oligonucleotides in a variety of *in vitro*

experimental situations to specifically degrade an mRNA of unknown function and thereby determine its physiologic function.

The oligonucleotides of the invention can be also used in clinical practice for any disease and against any target RNA for which antisense therapy is now known to be suitable or which is yet to be identified. Medical conditions for which antisense therapy is reported to be suitable includes Respiratory Syncytial Virus infection, WO 95/22553 by Kilkuskie, Influenza Virus infection, WO 94/23028, and malignancies, WO 94/08003. Further examples of clinical uses of antisense oligonucleotides are reviewed. in summary form, in Glaser, V., 1996, Genetic Engineering News 16, 1. Targets of antisense oligonucleotides under that are the subjects of clinical trials include protein kinase Cα, ICAM-1, c-raf kinase, p53, c-myb and the bcr/abl fusion gene found in chronic myelogenous leukemia.

5. EXAMPLES

5.1. EXPERIMENTAL CONDITIONS

The antisense activity of the oligonucleotides of the present invention are demonstrated using a test transient expression system which includes an mRNA encoding a luciferase protein that has been modified to include a test sequence derived from the *ras* gene. The specific antisense effects of an oligonucleotide can be measured by comparing the luciferase production of the test cells with the production of control cells having the same expression plasmid except for the absence of the ras-derived sequence. The oligonucleotides of the invention which were tested have the sequence: 5'-TTGCCCACACCGACGGCGCCCACCA-3' (SEQ ID NO: 1)

The details of the assay are as follows:

Plasmid Constructs. The plasmid used for the studies contained a portion of the ras gene sequence fused to luciferase (Monia, B.P., *et al.* J. Biol. Chem. 267:19954-19962 (1992)). The control luciferase plasmids did not contain the *ras* target sequence.

Cell Culture Assay. HeLa cells were grown to 40-90% confluence in DMEM/10% FBS, Supplemented with glutamine, penicillin and streptomycin on gelatin coated 24 well plates. The gelatin coating was necessary for cell to remain adherent during the transfections. Prior to transfection the cells were washed twice with PBS (containing magnesium and calcium). LIPOFECTIN™ was mixed gently and 6.6μl was added for each milliliter of reduced serum medium (OPTI-MEM™, Gibco/BRL, Gaithersberg, MD). Oligomers were added from 50-100μM concentrated stock to make a master mixture. The Opti-MEM/LIPOFECTIN/oligomer solution was added to the cells and incubated for 4 hours (~0.5 ml for one well of a 24 well plate).

A target transfection mixture was prepared by first diluting 5μl of lipofectin per ml of OPTI-MEM and mixing. Next 5μg of luciferase target and 5μg of CMV ß-galactosidase were added per milliliter of OPTI-MEM/LIPOFECTIN™ mixture. The transfection mixture was mixed gently and allowed to complex for about 15 minutes.. The master mixture reduced error by assuring that the control and experimental cells received the exact same cationic lipid/plasmid complex. The concentration of oligonucleotide in the culture medium was between 200 nM and 400 nM in all experiments. The oligonucleotide containing media was removed from the cells and replaced with growth media and incubated for an additional 9-18 hours. The cells were rinsed with calcium and magnesium free media and the media was removed. The plates were frozen at -70° C for >20 minutes and 100-300 μl of reporter lysis buffer (Promega, Madison WI) was added.

The cells were put through two more freeze thaw cycles, to assure complete lysis. Luciferase assays were performed according to the manufacture's instructions (Promega, Madison WI) and luminescence was detected with a 96 well luminometer (Packard, Meriden CT). ß-galactosidase assays were preformed (Galacton Plus, Tropix) according to manufactures instructions and detected on the Packard luminometer.

5.2. **EXPERIMENTAL RESULTS**

The results of the luciferase assays are presented in Table I. The results are reported as the percent specific inhibition as calculated by the formula 100 x (1- $(LUC_T/LUC_C)^{OLIGO}$ / $(LUC_T/LUC_C)^{NO\ OLIGO}$); wherein LUC_T and LUC_C are the luciferase levels found in the cells transfected with luciferase plasmids containing and lacking the ras gene insert (SEQ ID NO: 1); and the superscripts "Oligo" and "No Oligo" refer to the presence and absence of antisense oligonucleotides.

TABLE I

Oligo	Formula	Specific inhibition
	Controls ("C")	
C1	25Mo	26%
C2	25Ms	15%
C3	9Ds16Mo	15%
C4	9Do16MoInvT	0%
C5	9Dp16MoInvT	18%
C6	9Dp13Mo3Ms	14%
	Controls with all "S"	
S1	25Ds	93%
S2	16Ms8DsD	100%
S3	8Ms9Ds7MsM	97%
S4	9Ds15MsM	95%
	9Ds at 3' end ("3'I")	
3'I1	InvTMs15Mo9DsInvT	59%
3'I2	2Ms14Mo9DsInvT	57%
3'I3	4Ms12Mo9DsInvT	65%
	9Ds in Middle ("MI")	
MI1	5Ms3Mo9Ds4Mo3MsM	64%

MI2	2Ms6Mo9Ds7(MsMo)InvT	71%
MI3	3Ms6Mo9Ds6MoMsInvT	87%
	9Ds at 5'end ("5'I")	
5'I1	9Ds16MoInvT	83%
5'I2	9Ds15MoMsInvT	85%
5'I3	9Ds16MoBiotin	90%
5'I4	9Ds16Mp	91%
5'I5	9Ds14MoMpD	90%
5'I6	9Ds13Mo2MpD	94%
5'I7	9Ds12Mo3MpD	94%
5'I8	9Ds14MoMsD	93%
5'I9	9Ds13Mo2MsD	97%
5'I10	9Ds12Mo3MsD	95%

Key: M and D refer to 2'O-methyl- and 2'deoxy-ribonucleotides, respectively. The letters "O", "s" and "p" refer to phosphodiester, phosphorothioate diester, and P-ethoxy-phosphotriester linked nucleotides. "InvT" refers to a 3'→3' or 5'→5' linked thymidine located at the respective 3' or 5' end of the oligomer.

Table I shows the results of control oligos C1-C6, all phosphorothioate oligos S1-S4, and oligos of the invention having the RNase activating region at the 3' end (3'I1-3'I3), in the middle (MI1-MI3) and at the 5' end (5'I1-5'I10). Control oligos C1, C2, C5 and C6 showed low levels of specific inhibition because these oligos lacked an RNase H activating region. Oligos C3 and C4 were inactive because the 3' was unprotected and because native ssDNA was unstable, respectively. All phosphorothioate oligonucleotides (S1-S4) showed specific inhibitions that ranged between 93% and 100%, as did oligonucleotides 5'I6-5'I10, which have a 5'-located RNase H activating region and two or three 3' terminal 2'O-methyl modified P-ethoxy or phosphorothioate linked nucleotides (Mp and Ms, respectively). Lower levels of specific inhibition were observed when oligonucleotides with 3' and mid-located RNase H activating regions were employed or when suboptimal 3' protecting groups were present.

Although the oligonucleotides of the invention having 5' RNase activating regions achieved levels of specific inhibition which were comparable to those achieved by the uniform phosphorothioate oligonucleotides, the oligonucleotides of the invention were superior in that their use was associated with substantially less toxicity. Table II shows specific inhibition, the average metabolic activity as percent of no oligo control, as determined by MTS assay, and the percent viable cells, as determined by trypan blue exclusion for the conventional ("C"), all phosphorothioate ("S"), 3'I, MI and 5'I oligonucleotides, as well as for three species.

TABLE II

Oligo	% INH Luc	% of Control Metabolic Activity	% of Viable Cells
All "O" Oligos C1-C6	15%	94%	76%
All "S" Oligos S1-S4	96%	25%	21%
3'I (1-4)	60%	70%	61%
MI (1-3)	74%	77%	67%
5'I (1-10)	91%	71%	60%

The best oligos on the chart have high percentage values in all columns.

The results demonstrated that the oligonucleotides of the invention achieve levels of specific inhibition more than four times greater than conventional oligonucleotides while showing toxicity levels that were substantially lower than the phosphorothioate oligonucleotides. The optimal group, 5'I, showed specific inhibition that was comparable to the phosphorothioate oligonucleotides.

5.3. THE EFFECT OF THE LOCATION OF THE RNase H ACTIVATING REGION

The cause of lower specific activity observed for the 3'I and MI type oligonucleotides was investigated. One possibility was that the oxidation steps using 0.02 M I_2 caused the oxidation of the phosphorothioate linkages to phosphodiester, when phosphodiester linked nucleotides were added 5' to the phosphorothioate linkages. This was found to be the case. Comparison of oligonucleotides $9D_s15D_oD$ ("5'S") and $15D_o9D_sD$ ("3'S") oligonucleotides having the sequence of the test oligonucleotide by analytical HPLC analysis showed that about 85% of the 5'S oligonucleotides were fully thiolated, by contrast only 26% of the 3'S oligonucleotides were completely thiolated (36% were S-1, 24% S-2 and 14% S-3).

Table III shows the distribution of fully thiolated and mono, di and tri-oxidized by-products as a function of the position of the phosphorothiolated region of the oligonucleotide. Four thymidyl pentadodecamers were synthesized using 0.02 M I_2 as the oxidant for 15 nucleotides and a thiolating agent for nine nucleotides.

TABLE III

Ts	$[I_2]$	S	S-1	S-2	S-3
5'-9Ds15DoD03'	0.02M	96%	4%	-	-
5'-1Do9Ds14DoD-3'	0.02M	85%	15%	-	-
5'-8Do9Ds7DoD-3'	0.02M	41%	46%	12.5	0.5
5'-15Do9DsD-3'	0.02M	32%	43%	20%	5%
5'-15Do9DsD-3'	0.001M	78%	14%	8%	-

The results demonstrated that 96% of the 5'S oligonucleotides are fully thiolated and this percentage steadily decreased as the phosphorothioate region was exposed to more frequent oxidation reactions. When the oxidant concentration was reduced to 0.001M, 78% fully thiolated 3'S 25-T oligonucleotides and about 60% of oligonucleotides having the sequence of the SEQ ID NO: 1 were synthesized.

All publications and patents mentioned in the above specification are herein incorporated by reference. Various modifications and variations of the described method and system of the invention will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although the invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the above described modes for carrying out the invention which are obvious to those skilled in the field of molecular biology or related fields are intended to be within the scope of the following claims.

WE CLAIM:

1. A chimeric antisense oligonucleotide comprising: a 5'-terminus; a 3' terminus; and about 11 to about 59 5' to 3'-linked nucleotides independently selected from the group consisting of 2'-deoxyphosphorothioate nucleotides, 2'-modified phosphorothioate nucleotides, 2'-modified phosphodiester nucleotides, 2'-modified P-alkyloxyphosphotriester nucleotides, wherein:

a) said oligonucleotide incorporates an RNase H activating region of between about 3 and about 12 contiguous 2'-deoxyphosphorothioate-linked bases;

b) the 5' most 5' to 3' nucleotide linkage is a phosphorothioate or a P-alkyloxyphosphodiester linkage;

c) the 3' most 5' to 3' nucleotide linkage is a phosphorothioate or P-alkyloxyphosphodiester linkage or the 3' terminus is blocked; and

d) the oligonucleotide contains not more than 12 contiguous 2'-deoxyphosphorothioate linkages.

2. The oligonucleotide of claim 1, provided the 3' terminus is not blocked by a 3' to 3' phosphorothioate linked nucleotide.

3. The oligonucleotide of claim 1, in which the 3' terminus is blocked by a moiety comprising a 3' to 3' phosphorothioate linked nucleotide.

4. The oligonucleotide of claim 1, in which the 3' terminus is blocked by a moiety comprising a 3' to 3' phosphodiester linked nucleotide.

5. The oligonucleotide of claim 4, in which the 3' most 5' to 3' nucleotide linkage is a phosphorothioate linkage or a P-ethoxyphosphotriester linkage.

6. The oligonucleotide of claim 4, in which the 5' most 5' to 3' nucleotide linkage is a phosphorothioate linkage or a P-ethoxyphosphotriester linkage.

7. The oligonucleotide of claim 1, in which 2'-modified phosphorothioate nucleotides are present at both the 3' terminus and the 5' terminus.

8. The oligonucleotide of claim 1, in which the RNase H activating region is located at the 5' terminus.

9. The oligonucleotide of claim 8, in which the 3' most 5' to 3' nucleotide linkage is a phosphorothioate linkage or a P-ethoxyphosphotriester linkage.

10. The oligonucleotide of claim 9, in which the two 3' most 5'→3' internucleotide linkages are independently either a phosphorothioate linkage or a P-ethoxyphosphotriester linkage.

11. The oligonucleotide of claim 9, in which the RNase H activating region is contiguous with the 3' most 5'→3' nucleotide linkage.

12. The oligonucleotide of claim 11, in which the 2'-modified phosphodiester nucleotide is a 2'-methoxy or 2'-fluoro nucleotide.

13. The oligonucleotide of claim 11, which additionally comprises at least thirteen 2'-methoxy phosphodiester nucleotides.

14. The oligonucleotide of claim 11, having between 15 and 50 nucleotides.

15. The oligonucleotide of claim 14, which additionally comprises at least eight 2'-methoxy phosphodiester nucleotides.

16. The oligonucleotide of claim 14, which additionally comprises at least thirteen 2'-methoxy phosphodiester nucleotides.

17. The oligonucleotide of claim 1, in which the RNase H activating region comprises the 3' terminus.

18. The oligonucleotide of claim 1, in which the 2'-modified phosphodiester nucleotides are selected from the group consisting of 2'-fluoro and 2'-methoxy nucleotides.

19. The oligonucleotide of claim 4, in which the RNase H activating region is present at the 5' terminus followed by four to about forty 5' to 3' linked 2'-methoxy nucleotides, and the 3' terminus is blocked by a 3' to 3' phosphodiester linked deoxyribonucleotide.

20. A method of specifically cleaving an RNA in a cell containing RNase H which comprises administering an effective amount of an oligonucleotide complementary to the RNA, said oligonucleotide comprising: a 5' terminus; a 3' terminus; and about 11 to about 59 5' to 3'-linked nucleotides independently selected from the group consisting of 2'-deoxyphosphorothioate nucleotides, 2'-modified phosphorothioate nucleotides, 2'-modified phosphodiester nucleotides, 2'-modified P-alkyloxyphosphotriester nucleotides, wherein:

a) said oligonucleotide incorporates an RNase H activating region of between about 3 and about 12 contiguous phosphorothioate-linked 2'-deoxynucleotides;

b) the 5' most 5' to 3' nucleotide linkage is a phosphorothioate or a P-alkyloxyphosphodiester linkage;

c) the 3' most 5' to 3' nucleotide linkage is a phosphorothioate or P-alkyloxyphosphodiester linkage or the 3' terminus is blocked; and

d) the oligonucleotide contains not more than twelve contiguous phosphorothioate linked 2'-deoxynucleotides.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/17338

A. CLASSIFICATION OF SUBJECT MATTER
IPC(6) : C12Q 1/68; C07H 21/04
US CL : 435/6,91.1; 536/24.5
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 435/6,91.1; 536/24.5

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
APS, DIALOG: BIOSIS, MEDLINE, CAS, Derwent Biotechnology Abstracts

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Mesmaeker et al. Backbone modifications in oligonucleotides and peptide nucleic acid systems. Current Opinion in Structural Biology. 1995. Vol. 5. pages 343-355, see entire document.	1-20

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
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